US Environmental Protection Agency
Office of Pesticide Programs

Office of Pesticide Programs
Microbiology Laboratory
Environmental Science Center, Ft. Meade, MD

Standard Operating Procedure for
Tuberculocidal Activity of Disinfectants:
In vitro Test for Determining Tuberculocidal Activity

SOP Number: MB-07-06

Date Revised: 12-06-12

SOP Number	MB-07-06
Title	Tuberculocidal Activity of Disinfectants: *In vitro* Test for Determining Tuberculocidal Activity
Scope	Describes the methodology used to determine tuberculocidal activity of disinfectants against *Mycobacterium bovis* (BCG) on hard surfaces (see 15.1).
Application	For product evaluations under the Antimicrobial Testing Program (ATP), a study protocol is developed which identifies the specific test conditions for a product sample such as contact time, dilutions, neutralizers, etc.

	Approval Date
SOP Developer:	_____ Print Name: _____
SOP Reviewer	_____ Print Name: _____
Quality Assurance Unit	_____ Print Name: _____
Branch Chief	_____ Print Name: _____

Date SOP issued:	
Controlled copy number:	
Date SOP withdrawn:	

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1. Definitions	Additional abbreviations/definitions are provided in the text. Carrier Set = One "carrier set" is defined as the primary MPB tube containing the carrier and the two additional subculture media tubes (e.g., M7H9 broth, Kirchners medium, or TB broth) inoculated from the carrier's corresponding neutralizer tube. There are 10 carrier sets per disinfectant tested.
2. Health and Safety	Follow procedures specified in SOP MB-01, Laboratory Biosafety. The Study Director and/or lead analyst should consult the Material Safety Data Sheet for specific hazards associated with products.
3. Personnel Qualifications and Training	Refer to SOP ADM-04, OPP Microbiology Laboratory Training.
4. Instrument Calibration	Refer to SOPs EQ-01, EQ-02, EQ-03, EQ-04 and EQ-05 for details on method and frequency of calibration.
5. Sample Handling and Storage	Refer to SOP MB-22, Disinfectant Sample Preparation, and SOP COC-01, Chain of Custody Procedures.
6. Quality Control	For quality control purposes, the required information is documented on the appropriate form(s) (see section 14).
7. Interferences	Transferring the inoculated carrier into the tube containing the disinfectant is a critical, technique-sensitive step. False positives can result from transfer of test microbe to sides of tubes due to inadvertent contact.
8. Non-conforming Data	1. Sterility and/or viability controls failed to yield expected results. 2. The mean log density for control carriers falls outside the specified range. Note: The prescribed minimum and maximum carrier counts also account for the addition of 5% organic soil to the inoculum. a. The mean *TestLD* must be at least 4.0 (corresponding to a geometric mean density of 1.0×10^4) and not above 6.0 (corresponding to a geometric mean density of 1.0×10^6); a mean *TestLD* below 4.0 or above 6.0 invalidates the test, except for two retesting scenarios (outlined in the study protocol). 3. Management of non-conforming data will be specified in the study protocol; procedures will be consistent with SOP ADM-07, Non-Conformance Reports.
9. Data Management	Data will be archived per SOP ADM-03, Records and Archives.
10. Cautions	1. There are time sensitive steps in this procedure including the use periods

	of the inoculated carriers and the test chemical. 2. Verify the volume of dilution blanks, neutralizer tubes, and subculture tubes in advance and adjust accordingly.
11. Special Apparatus and Materials	1. Culture media. a. *Modified Proskauer-Beck medium*. Dissolve 2.5 g KH_2PO_4, 5.0 g asparagine, 0.6 g $MgSO_4 \times 7H_2O$, 2.5 g magnesium citrate, 20.0 mL glycerol, 0.0046 g $FeCl_3$, and 0.001 g $ZnSO_4 \times 7H_2O$ in 1 L H_2O. Adjust to pH 7.2-7.4 with 1 N NaOH. Filter through Whatman No. 4[1] (or equivalent) filter paper, place 20 mL portions in separate 25 × 150 mm tubes, and steam sterilize 20 min at 121°C. Use this broth for propagating test cultures and for recovery of test organism from treated carriers. b. *Middlebrook 7H9 agar* (dehydrated M7H9 medium + agar). Dissolve 4.7 g in 900 mL H_2O containing 2 mL glycerol and 15.0 g agar. Heat to boiling to dissolve completely. Steam sterilize 15 min at 121°C. Cool sterile medium to 45°C, add 100 mL Middlebrook ADC Enrichment under aseptic conditions, and mix thoroughly. Distribute in 20 mL portions in sterile 25 × 150 mm screw-capped tubes and slant or dispense a minimum of 30 mL into sterile Petri plates. Use slants to maintain stock culture and plates for inoculum isolation and enumeration. c. *Middlebrook 7H11 agar (dehydrated M7H11 medium)*. Dissolve 21 g dehydrated M7H11 agar medium in 900 mL H_2O containing 5 mL glycerol. Swirl to obtain a smooth suspension; boil if necessary to completely dissolve the powder. Steam sterilize 15 min at 121°C. Cool sterile medium to 50-55°C, add 100 mL OADC enrichment under aseptic conditions, and mix thoroughly. Distribute in 20 mL portions in sterile 25 × 150 mm screw-capped tubes and slant or dispense a minimum of 30 mL into sterile Petri plates. Alternatively, pre-made M7H11 agar plates may be purchased. Use slants to maintain stock culture and plates for inoculum isolation and enumeration. d. *Middlebrook 7H9 broth (dehydrated M7H9 medium)*. Dissolve 4.7 g in 900 mL H_2O containing 2 mL glycerol and 1.0 g Bacto agar. Heat to boiling to dissolve completely. Steam sterilize 15 min at 121°C. Cool sterile medium to 45°C, add 100 mL Middlebrook ADC Enrichment under aseptic conditions and mix thoroughly. Distribute in 20 mL portions in sterile 25 × 150 mm tubes. Use for recovery of

[1] MLB proposed revision, not currently in AOAC Method 965.12.

test organism from treated carriers.

e. *Kirchners medium*. Dissolve 5 g asparagine, 2.5 g sodium citrate, 0.6 g magnesium sulfate (heptahydrate), 2.5 g monopotassium phosphate, and 1.5 g dipotassium phosphate, in 900 mL H_2O containing 20 mL glycerol and 1.0 g Bacto agar. Heat to boiling to dissolve completely. Steam sterilize 15 min at 121°C. Cool sterile medium to 45°C, add 100 mL Middlebrook ADC Enrichment under aseptic conditions, and mix thoroughly. Distribute in 20 mL portions in sterile 25 × 150 mm tubes. Use for recovery of test organism from treated carriers.

f. *TB broth base*. Dissolve 2.0 g yeast extract, 2.0 g proteose peptone No. 3, 2.0 g casitone, 1.0 g potassium phosphate monobasic, 2.5 g sodium phosphate dibasic, 1.5 g sodium citrate, and 0.6 g magnesium sulfate (heptahydrate) in 900 mL H_2O containing 50 mL glycerol and 1.0 g Bacto-agar. Heat to boiling to dissolve completely. Steam sterilize 15 min at 121°C. Cool sterile medium to 45°C, add 100 mL Dubos Medium Serum under aseptic conditions, and mix thoroughly. Distribute in 20 mL portions in sterile 25 × 150 mm tubes. Use for recovery of test organism from treated carriers.

2. Test organism.

a. *Mycobacterium bovis* (BCG) (Organon Teknika Corp., Durham, NC, USA, or equivalent). For stock culture, streak inoculate M7H9 or M7H11 agar slants. Incubate 15-20 days at 36 ± 1°C. Following incubation, maintain at 2-5°C for up to 6 weeks.[2]

3. Reagents

a. *Sterile water*.—Use reagent-grade water free of substances that interfere with analytical methods. Any method of preparation of reagent-grade water is acceptable provided that the requisite quality can be met. See Standard Methods for the Examination of Water and Wastewater and SOP QC-01, Quality Assurance of Purified Water for details on reagent-grade water.

b. *0.1% polysorbate 80 in saline*. Add 0.1 mL polysorbate 80 to 100 mL sterile 0.85% aqueous saline (sodium chloride) solution, filter sterilize. Used in test culture preparation.

c. *Octylphenoxypolyethoxyethanol nonionic surfactant* (e.g.Triton X-100).

[2] MLB proposed revision, not currently in AOAC Method 965.12.

	4. Apparatus. a. *Specialized glassware*. For disinfectant, use autoclavable 25 × 100 mm tubes (Bellco Glass Inc., or equivalent). For glassware used to prepare test chemical, refer to SOP MB-22. b. *Tissue grinder*. Kimble glass tissue grinder (885300-0015).[3] c. *Recirculating chiller unit*. For maintaining specified temperature of the test chemical. d. *Inoculating loop*. For culture inoculation, 1 µL sterile disposable loops (Fisher Scientific).[4] For culture harvest, 95% platinum, 3.5% rhodium alloy, 18 or 19 gauge, 4 mm loop with 75 mm shank (Baxter Scientific Products) or equivalent or disposable loops. e. *Wire hook*. For carrier transfer. Make 3-5 mm bend (approximately 60°) at end of suitable platinum or platinum alloy wire, No. 23 B&S gauge, in appropriate holder (Johnson Matthey Inc., or equivalent). f. *Carriers*. "Penicylinders," porcelain, 8 ± 1 mm outer diameter, 6 ±1 mm inner diameter, 10 ± 1 mm long (CeramTec Ceramic; Cat. No. LP15819 0645). Use only carriers that passed physical screening; refer to SOP MB-03, Screening of Stainless Steel Cylinders, Porcelain Cylinders and Glass Slide Carriers Used in Disinfectant Efficacy Testing. g. *Timer*. Any certified timer that can display time in seconds. h. *Spectrophotometer*. Calibrated; for preparing standardized test culture. i. *Sonicator (ultrasonic cleaner).* For conducting control carrier counts. If necessary, validate by placing broth culture into sonicator for 10 min. Bacterial plate count must equal an unexposed control.[5] j. *Semimicrocuvette with cap.* For measuring percent transmittance. k. *TB Stain Kit.* For presumptive identification of test microbe.
12. Procedure and Analysis	An assessment of media quality (performance) is necessary to ensure the validity of the tuberculocidal efficacy results. The media assessment may be conducted in advance of or concurrently with efficacy testing; refer to SOP MB-10, Media and Reagents Used in Microbiological Assays Including Performance Assessment and Sterility Verification.

[3] MLB proposed revision, not currently in AOAC Method 965.12.
[4] MLB proposed revision, not currently in AOAC Method 965.12.
[5] MLB proposed revision, not currently in AOAC Method 965.12.

12.1 Test Culture Preparation	Refer to SOP MB-02 for the test microbe culture transfer notation. a. Initiate test culture by inoculating a sufficient number of 25 × 150 mm tubes containing 20 mL MPB (approximately 10) from stock culture slant(s) (M7H9 or M7H11 agar slants) by transferring 1-2 1 µL loopfuls[6] from the stock culture onto the surface of the broth. Record all transfers on the Organism Culture Tracking Form (culture notation = –SL, indicating a transfer from slant to liquid). Note: Over-inoculation of MPB may lead to reduced viability due to excessive growth after 21 ± 2 days; the resulting carrier counts may be negatively impacted. b. Incubate the tubes 21 ± 2 days undisturbed at 36 ± 1°C in a slanted position to increase surface area. c. On the test day: Using a transfer loop, transfer culture to a heat-sterilized glass tissue grinder, add 1.0 mL 0.1% polysorbate 80 in saline solution, grind to break up large clumps or aggregates of the test organism. d. Dilute the homogenized culture with 9 mL MPB broth and transfer the suspension from the tissue grinder to a sterile test tube. Harvest and homogenize culture from multiple MPB broth tubes.[7] Note: Growth from multiple tubes may be harvested and combined to prepare the concentrated culture prior to standardization. e. Allow the suspension to settle for 10-15 min. f. Remove the upper portion of each culture, leaving behind any debris or clumps, and transfer to a sterile flask; pool cultures in the flask and swirl to mix. g. Dilute the pooled culture with MPB broth to achieve 20 ± 1% T at 650 nm. Use a semimicrocuvette with cap while measuring transmittance. h. If an organic soil load is specified in the test parameters for the product test, the appropriate amount of organic soil is added to the pooled test culture prior to the inoculation of carriers. Swirl to mix. i. Use standardized culture to inoculate porcelain cylinders.
12.2 Carrier Inoculation	Inoculate approximately 20 carriers; 10 carriers are required for testing, 3 for control carrier counts, and 3 for viability controls.

[6] MLB proposed revision, not currently in AOAC Method 965.12.
[7] MLB proposed revision, not currently in AOAC Method 965.12.

	a. Inoculate sets of 10 sterile carriers with approximately 15-20 mL standardized test culture in 25 × 150 mm test tubes. b. The test culture must completely cover the carriers. If a carrier is not covered, gently shake the tube, or reposition the carrier within the tube with a sterile wire hook. Be sure to inoculate a sufficient number of carriers for the test. c. After 15 ± 1 min contact period, remove cylinders using flamed wire hook and shake carriers vigorously against side of the tube to remove excess culture. (Alternatively, drain the inoculum from the carriers with a pipette; avoid direct contact of the carrier with the tip of the pipette. Briefly tap each carrier against the side of the tube to remove excess culture.)[8] Place carriers on end in vertical position in sterile Petri dishes matted with 2 layers of Whatman No. 2 (or equivalent) filter paper, making sure that carriers do not touch to prevent improper drying. Place no more than 12 carriers in a Petri dish. d. Carriers that touch or fall over cannot be used for testing and must be removed, cleaned, and sterilized. e. Once all of the carriers have been transferred, cover and place in incubator at 36 ± 1°C, and let dry 30 ± 2 min. Record the time on the AOAC Tuberculocidal Test Processing Sheet (see section 14). f. Use inoculated carriers for testing within 2 h of drying.
12.3 Enumeration of bacterial inocula (carrier counts)	a. After inoculated carriers have dried, randomly select 3 inoculated carriers for assay. Assay 1 carrier immediately prior to conducting the efficacy test and 2 carriers following the test. b. Place each inoculated carrier into a tube containing 10 mL of MPB broth and sonicate in an ultrasonic cleaner for 10 min. Record the time of sonication on the AOAC Tuberculocidal Test Processing Sheet (see section 14). c. For sonication, place tubes into an appropriately sized glass beaker with tap water to the level of the MPB broth in the tubes. Place the beaker in an ultrasonic cleaner so that the water level in the beaker is even with the water level fill-line on the tank. Fill the tank with tap water to the water level fill-line. Hold the beaker so that it does not touch the bottom of the tank and all 3 liquid levels (inside the test tubes, inside the beaker, and inside the tank) are approximately the same.

[8] MLB proposed revision, not currently in AOAC Method 965.12.

	d. After sonication, briefly mix each tube on a vortex mixer and make serial ten-fold dilutions in 9 mL phosphate buffered dilution water. If the serial dilutions are not made and plated immediately, the sonicated tubes are kept at 2-5°C until this step can be done; however perform dilution and plating within 2 h of sonicaiton. e. Briefly mix each serial dilution tube prior to plating. Plate 0.1 mL aliquots of appropriate dilutions in duplicate on M7H9 or M7H11 using surface spread plating. Dilutions 10^{-1} through 10^{-3} should produce plates with CFU in the appropriate range. Spread inoculum evenly over the surface of the agar. Plates must be dry prior to incubation. f. Incubate plates (inverted) concurrently with the efficacy test subculture tubes at 36 ± 1°C for 17-21 days. g. Count colonies. Plates that have colony counts over 300 will be reported as TNTC. Record counts on the AOAC Tuberculocidal Test Carrier Counts Form (see section 14). See section 13 for data analysis.
12.4 Disinfectant Sample Preparation	a. Prepare disinfectant sample per SOP MB-22. b. Equilibrate the water bath and allow it to come to 20 ± 1ºC or the temperature specified (± 1ºC). Prepare the disinfectant dilution within 3 hours of performing the assay unless test parameters specify otherwise. Record the time of disinfectant preparation on the AOAC Tuberculocidal Test Processing Sheet (see section 14). c. Dispense 10 mL aliquots of the disinfectant into 25 × 100 mm test tubes, one tube per carrier. Place tubes in the equilibrated water bath for approximately 10 min to allow disinfectant to come to specified temperature. Record the temperature of the water bath and recirculating chiller before and after testing on the AOAC Tuberculocidal Test Information Sheet (see section 14).
12.5 Test Procedure	a. Sequentially transfer carriers from Petri dish to test tubes containing disinfectant at appropriate intervals (e.g., 1 min intervals). Record timed transfer activities on the AOAC Tuberculocidal Time Recording Sheet for Carrier Transfers (see section 14). b. Add one carrier per tube. For a contact time of 10 min, the carrier must be deposited in the tube within ± 5 s of the prescribed drop time. c. Using alternating hooks, sterilize the hook and allow it to cool after each carrier transfer. When lowering the carrier into the disinfectant

tubes, neither the carrier nor the wire hook should touch the interior sides of the tube. If the interior sides of the tube are touched, repeat the carrier.

d. Following the exposure time, sequentially transfer the carriers into neutralizer tubes using a sterile hook. Drain excess disinfectant from the carrier prior to transfer.

e. Shake tube containing carrier in neutralizer thoroughly; transfer the carrier to the tube containing 20 mL MPB broth within 5-10 minutes.[9] Sterilize hook after each carrier transfer. Avoid contact of the carrier to the interior of the tube during transfer.

f. Once all carriers have been transferred to the MPB broth tubes, sequentially transfer 2 mL aliquots from each neutralizer tube into 2 additional subculture media, M7H9 broth, Kirchners medium, or TB broth, as specified. This portion of the assay is not timed, but the aliquots should be sequentially transferred to the subculture media within approximately 30 ± 5 min. Repeat this with each tube of neutralizer. Shake each subculture tube thoroughly. Slightly loosen caps of growth media prior to incubation.[10]

g. Incubate 60 days at 36 ± 1°C.

h. Report results as + (growth) or 0 (no growth).

i. Record results at 60 days. If the 60th day of incubation falls on a weekend or holiday, record the results on the first workday following the 60th day of incubation.

   i. Tubes may be monitored beginning at day 21 for evidence of typical mycobacterial growth. If multiple tubes show significant growth prior to the 60th day, confirmatory tests (e.g., acid fast staining and streak isolation) may be initiated prior to day 60. If the results of the confirmatory test are indicative of *M. bovis* (BCG), the results may be recorded at that point to expedite the reporting process.

   ii. Provide justification when recording results on days other than 60 in the comments section of the AOAC Tuberculocidal Test Results Sheet (see section 14).

j. If no growth or occasional growth (insufficient for confirmatory tests) occurs within a set of tubes, incubate the set an additional 30 days and record the results. Growth should be checked by using

[9] MLB proposed revision, not currently in AOAC Method 965.12.
[10] MLB proposed revision, not currently in AOAC Method 965.12.

		standard confirmatory procedures (e.g., acid fast staining and growth on M7H9 or M7H11 agar) to ensure that no contamination is present. k. Record results at 90 days. If the 90th day of incubation falls on a weekend or holiday, record the results on the first workday following the 90th day of incubation. Recording of results beyond the 90th day should be notated in the Comments section on the AOAC Tuberculocidal Test Results Sheet (see section 14).
12.6	Sterility and viability controls	a. Sterility controls. Place one sterile, uninoculated carrier into a tube of MPB broth. In addition, incubate 1 tube of each subculture medium with 2 mL sterile neutralizer for quality control purposes. Shake each tube thoroughly and incubate all tubes with the efficacy test. Report results as + (growth) or 0 (no growth) as determined by presence or absence of turbidity or presence of culture growth. Growth should not occur in any tube. Record results on the AOAC Tuberculocidal Test Results Sheet (see section 14). b. Viability controls. On the day of testing, place a dried inoculated carrier into a tube of MPB broth and a tube of each subculture media. Incubate tubes as in the efficacy test. Report results as + (growth) or 0 (no growth) as determined by presence or absence of turbidity or presence of culture growth. Growth should occur in all tubes. Record results on the AOAC Tuberculocidal Test Results Sheet (see section 14).
12.7	Test Microbe Identification	a. Presumptively confirm at least one positive subculture tube for each carrier set with growth. The maximum number of tubes subjected to confirmatory tests per disinfectant tested is 10. b. If more than one subculture tube for a carrier set is positive, confirm a minimum of one tube using acid fast staining and isolation on selective media (M7H9 or M7H11 agar plates). c. If the MPB in the set is positive, it is the representative subculture tube used for identification. If MPB is not positive, any of the other subculture media may be used for identification. d. If growth is observed in only one carrier set, then all subculture tubes showing growth for that carrier are subject to confirmatory tests. e. Growth for acid fast staining is taken from the selected positive tubes on the day that results are read. Acid fast rods are typical for *M. bovis* (BCG). The acid fast staining results should be read promptly prior to assigning a + or 0 to the results. If acid fast rods are observed from the selected tubes then a + is assigned to the results. If no cells are observed for the acid fast stain then a 0 is applied to

	the results. f. In addition, streak isolate growth from positive tubes on M7H9 or M7H11 agar and incubate for 17-21 days at 36 ± 1ºC. g. Following the 17-21 day incubation period, evaluate the colony morphology on M7H9 or M7H11 agar. *M. bovis* (BCG) typically appears as colorless to buff-colored, raised, rough growth on M7H9 and M7H11 agar (see Attachment 1). h. If a satisfactory smear cannot be obtained directly from the tube, take the smear for acid fast staining from the 17-21 day old M7H9 or M7H11 agar plate that was inoculated with the growth from the tube. i. In the event that no cells were observed with acid fast staining initially but typical growth was observed on the M7H9 or M7H11, correct the 0 to read + on the test sheet. An entry error will be noted in the comments section of the AOAC Tuberculocidal Test Results Sheet (see section 14). j. Record results on the AOAC Tuberculocidal Test Microbe Confirmation Sheet (see section 14).
13. Data Analysis/ Calculations	Calculations will be computed using a Microsoft Excel spreadsheet (see section 14). Both electronic and hard copies of the spreadsheet will be retained. Counts from 0 through 300 and their associated dilutions will be included in the calculations.
14. Forms and Data Sheets	1. Attachment 1: Typical Growth Characteristics of strains of *M. bovis* (BCG) 2. Attachment 2: Culture Initiation and Stock Culture Generation for *Mycobacterium bovis* (BCG) 3. Test Sheets. Test sheets are stored separately from the SOP under the following file names: Physical Screening of Carriers Record — MB-07-06_F1.docx AOAC Tuberculocidal Activity of Disinfectants Test: Time Recording Sheet for Carrier Transfers — MB-07-06_F2.docx AOAC Tuberculocidal Activity of Disinfectants Test: Test Information Sheet — MB-07-06_F3.docx AOAC Tuberculocidal Activity of Disinfectants Test: Results Sheet — MB-07-06_F4.docx AOAC Tuberculocidal Activity of Disinfectants Test: Test Microbe Confirmation Sheet — MB-07-06_F5.docx

	Organism Culture Tracking Form for *Mycobacterium bovis* (BCG)	MB-07-06_F6.docx
	Test Microbe Confirmation Sheet (Quality Control)	MB-07-06_F7.docx
	AOAC Tuberculocidal Activity of Disinfectants Test Carrier Counts Form	MB-07-06_F8.docx
	AOAC Tuberculocidal Activity of Disinfectants Test Processing Sheet	MB-07-06_F9.docx
	Carrier Count Spreadsheet (MS Excel): Carrier Count Template_CTB_v2	MB-07-06_F10.xlsx
15. References	1. Official Methods of Analysis. 2012. 18th Ed., AOAC INTERNATIONAL, Gaithersburg, MD, (Method 965.12 In vitro Test for Determining Tuberculocidal Activity). 2. Standard Methods for the Examination of Water and Wastewater. 2005. 21st Ed., American Public Health Association, Washington, D.C. 3. Holt, J., Krieg, N., Sneath, P., Staley, J., and Williams, S. eds. 1994. Bergey's Manual of Determinative Bacteriology, 9th Edition. Williams & Wilkins, Baltimore, MD. 4. Sneath, P., Mair, N., Sharpe, M.E., and Holt, J. eds. 1986. Bergey's Manual of Systematic Bacteriology. Volume 2. Williams & Wilkins, Baltimore, MD. 5. Package Insert – TB Stain Kits and Reagents. Becton, Dickinson and Company. Part no. 8820201JAA. Revision 03/2011.	

Attachment 1

Typical Growth Characteristics of strains of *M. bovis* (BCG) (see ref. 15.3 and 15.4)

	M. bovis (BCG)*
Gram stain reaction	weakly (+)
Acid Fast stain reaction	(+)
Typical Growth Characteristics on Solid Media	
Middlebrook 7H9	rough, raised, thick colonies with a nodular or wrinkled surface and an irregular thin margin, off-white to faint buff, or even yellow
Typical Microscopic Characteristics	
Cell dimensions	0.3-0.6 µm in diameter by 1-4 µm in length*
Cell appearance	rods, straight or slightly curved, occurring singly and in occasional threads

*After 15-20 days

Attachment 2

Culture Initiation and Stock Culture Generation for *Mycobacterium bovis* (BCG)

A1. Culture initiation. Refer to SOP MB-02 for establishment of the organism control number.

a. Obtain lyophilized culture of *M. bovis* (BCG).

b. Reconstitute the lyophilized culture with ~1 mL of sterile DI water. Inoculate two M7H9 or M7H11 agar plates by streaking for isolation.

c. Add ~0.2 mL of the rehydrated culture to each of 4 tubes of MPB.

d. Incubate the M7H9 or M7H11 agar plates and MPB broth tubes for 15 to 20 days at 36 ± 1ºC or until there is sufficient growth. Incubate MPB broth tubes in a slanted position.

A2. Culture maintenance.

a. Confirm the identity of the streak isolation plates and acid fast stain (see Attachment 1 for colony morphology and section 15.5 for acid fast staining). Afterwards, use the 15-20 day old MPB broth cultures (from section A1) to initiate stock cultures.

b. Streak M7H9 or M7H11 agar slants (stock slants) using 1-4 tubes of MPB broth cultures of *M. bovis* (BCG). Based on anticipated use, streak approximately 10-20 stock slants.

c. Incubate the new stock transfers for 15-20 days at 36 ± 1ºC. Store at 2-5ºC.

d. Every 6 weeks (42 days), generate an additional 10-20 M7H9 or M7H11 slants. Inoculate new M7H9 or M7H11 slants by streaking a loopful of *M. bovis* (BCG) growth from an established tube to each of the 10-20 tubes. Perform QC of stock cultures per section A3.

e. Incubate the stock culture slants at 36 ± 1ºC for 15 to 20 days. Following incubation, maintain stock cultures at 2-5ºC for up to 6 weeks.[11]

A3. QC of stock cultures

a. Up to every 6 weeks (42 days), streak a loopful of growth for isolation from the existing M7H9 or M7H11 stock slant used to inoculate new agar slants on a plate of M7H9 or M7H11 agar. Incubate the plate for 17-21 days at 36 ± 1ºC.

b. Following the incubation period, record the colony morphology as observed on the M7H9 or M7H11 plate. See Attachment 1 for details on cell and colony morphology and stain reactions.

c. Perform an acid fast stain (refer to 15.5) from growth taken from the M7H9 or M7H11 streak isolation plate according to the manufacturer's instructions. Observe

[11] MLB proposed revision, not currently in AOAC Method 965.12.

the acid fast reaction by using brightfield microscopy at 1000X magnification (oil immersion).

d. Record observations on the Test Microbe Confirmation Sheet (Quality Control) (see section 14).